TITLE OF THE INVENTION

INFECTIOUS ETIOLOGIC AGENT DETECTION PROBE AND PROBE SET, CARRIER, AND GENETIC SCREENING METHOD

FIELD OF THE INVENTION

The present invention relates to detection and/or identification of an infectious etiologic agent as an etiologic agent of an infectious disease and, more particularly, to a probe and probe set originated in an infectious etiologic agent, a carrier, and a genetic screening method, which are useful for detection and identification of the etiologic agent of an infectious disease.

The present invention also relates to a PCR amplification process of an infectious etiologic agent, which is suitable for detection and/or identification of an infectious etiologic agent.

BACKGROUND OF THE INVENTION

In recent years, gene expression analysis using DNA chips (also referred to as DNA microarrays hereinafter) is done in various fields including drug development. Different specimen DNAs are made to react with a DNA microarray in which various kinds of gene sets (probes) are arranged. Gene dosages which exist in the respective specimens are compared. Genes which

are present in high dosages (the expression amounts are large) or inactive genes (the expression amounts are small) at each stage are classified and analyzed in association with functions.

An example is an infectious etiologic agent test. In Japanese Patent Laid-Open No. 2001-299396, Ezaki et al have proposed a microorganism identification method using, as a DNA probe, a DNA chip on which chromosome DNAs are immobilized. According to this method, a plurality of chromosome DNAs originated in a plurality of known microorganisms with different GC contents are made to react with chromosome DNAs originated in an unknown microorganism in a specimen. When the resultant hybridization complex is detected, the unknown microorganism in the specimen can be detected.

As probes used for DNA chips for infectious etiologic agent tests, Ono et al have proposed a bacterial detection probe using restriction enzyme fragments in Japanese Patent Laid-Open No. 6-133798, a Pseudomonas aeruginosa detection probe in Japanese Patent Laid-Open No. 10-304896, and a detection probe using restriction enzyme fragments of Escherichia coli, klebsiella pneumoniae, and Enterobacter cloacae in Japanese Patent Laid-Open No. 10-304897.

As a microarray, for example, a microarray using stamping called a Stanford method is known. For example, DNA chips on which cDNA fragments of known

genes of human origin, which are related to cancers, are applied by spotting or stamping and chips prepared by bonding cDNA fragments of 1,000 kinds of known genes of human origin to slide glasses are commercially available from TAKARA SHUZO.

On the other hand, a chip available from Affymetrix is prepared by designing an oligonucleotide probe set on the basis of the known gene cDNAs, and probes are laid out by synthesis on a substrate. Oligoprobes are laid out on one chip at a high density so that the expression levels of 10,000 or more genes can be analyzed at once.

However, the DNA chips of the prior arts described above use DNA probes such as chromosome DNAs or restriction enzyme fragments. DNAs directly extracted from microorganisms are used as materials. For this reason, the chips can hardly be mass-prepared at a time and are not suitable for clinical diagnosis. For application to clinical diagnosis, mass production of inexpensive and uniform DNA chips is necessary. For this purpose, mass preparation of uniform DNAs as probe solutions is essential. However, mass preparation of DNA probes is impossible. Even for DNA probes, when PCR amplification reaction is used, the number of DNAs can gradually be increased. However, mass preparation at a time using the PCR reaction is difficult, and the DNA chips are difficult to use for clinical diagnosis.

In addition, since the base length of a DNA probe is large, it is difficult to identify one species in similar species. Such a DNA probe is therefore not suitable for, e.g., infection detection. In treating an infection, the species must be specified, and antibiotic drugs corresponding to it must be selected and administered. For this purpose, an infection detection probe is required to have a function capable of detecting a species while discriminating similar species, although bacteria belonging to the same species need not accurately be discriminated (that is, bacteria in the same species can be detected all together). However, in, e.g., the DNA chip using restriction enzyme fragments of Escherichia coli, klebsiella pneumoniae, and Enterobacter cloacae, which is disclosed in Japanese Patent Laid-Open No. 10-304897, cross reaction occurs between the three species because of the large base length of the probe. Since similar species cannot individually be discriminated, the DNA chip can hardly be used for infection detection.

As an application purpose of microarrays, infectious etiologic agent tests have received a great deal of attention. Some probe sets aiming at testing infectious etiologic agents have also been proposed.

As an important point of bacterial tests using microarrays, detection must be possible even when the

number of infectious etiologic agents is small. To do this, it is effective to amplify specific parts in the base sequences of the DNAs of infectious etiologic agents by, e.g., PCR reaction using primers. For example, a 16s rRNA gene arrangement contains a sequence unique to the species in the information of about 1,700 base pairs. When the sequence is used, classification can be done to some extent. In detecting/identifying bacteria, 16s rRNA parts in the DNA base sequences of bacteria are preferably used. Hence, it is demanded to amplify the 16s rRNA parts.

For various kinds of bacteria, however, the gene arrangements are only partially clarified, and the 16s rRNAs are not totally known. For this reason, it is not easy to design primers for PCR amplification reaction.

SUMMARY OF THE INVENTION

The present invention has been made in consideration of the above situation, and has as its object to provide an infection detection probe which allows mass preparation at a time and identification of a species in similar species.

More specifically, it is an object of the present invention to provide an infection detection probe which can suitably be used to classify a plurality of kinds of etiologic agents of an infection on the basis of the

species.

It is another object of the present invention to provide a probe set which also considers the stability of a hybrid body between an infection detection probe and a specimen so that the difference between similar species can accurately be evaluated on a DNA chip.

It is still another object of the present invention to provide a carrier on which the infection detection probe is immobilized to make the infection detection probe react with the specimen.

It is still another object of the present invention to provide a carrier on which the infection detection probes are chemically immobilized so that the infection detection probes are stably immobilized on the carrier, and a detection result with high reproducibility can be obtained in the process of reaction with a specimen solution.

It is still another object of the present invention to provide a PCR reaction primer which amplifies the 16s rRNAs of an etiologic agent in a specimen in order to detect and/or identify an infectious etiologic agent.

It is still another object of the present invention to provide a primer set which can commonly be used for a plurality of species and effectively amplify the 16s rRNAs of an etiologic agent even when the species is unknown.

It is still another object of the present invention to provide a primer set which can amplify the 16s rRNAs of a plurality of kinds of etiologic agents under the same PCR conditions.

The present invention provides a primer set characterized by amplifying all species without amplifying genes originated in human genomes by causing PCR reaction for a human blood specimen by using all the primer sets simultaneously. More specifically, a primer set having a sequence which is different from the base sequence of human genome genes by three or more bases is proposed.

Other features and advantages of the present invention will be apparent from the following description.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

A preferred embodiment of the present invention will now be described in.

In the following embodiment, an oligonucleotide probe used to identify the etiologic agent of an infection and, more specifically, a probe used to detect one or some of Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens, Streptococcus pneumoniae, Haemophilus influenzae, Enterobacter cloacae, and Enterococcus

faecalis will be described. That is, a nucleic acid probe or nucleic acid probe set, which is used to detect rRNA gene arrangements in the genes of the above 10 infectious etiologic agents in proper quantities, is disclosed.

According to this embodiment, the oligonucleotide probe to be reacted with a specimen solution containing the nucleic acid sequence of the genes of the infectious etiologic agents contains one base sequence which belongs to one of the first group (SEQ ID Nos. 1 to 14 in the attached sequence table) shown in Table 1, the second group (SEQ ID Nos. 15 to 24) shown in Table 2, the third group (SEQ ID Nos. 25 to 36) shown in Table 3, the fourth group (SEQ ID Nos. 37 to 47) shown in Table 4, the fifth group (SEQ ID Nos. 48 to 57) shown in Table 5, the sixth group (SEQ ID Nos. 58 to 68) shown in Table 6, the seventh group (SEQ ID Nos. 69 to 77) shown in Table 7, the eighth group (SEQ ID Nos. 78 to 85) shown in Table 8, the ninth group (SEQ ID Nos. 86 to 97) shown in Table 9, and the 10th group (SEQ ID Nos. 98 to 106) shown in Table 10 (to be described later). An oligonucleotide probe having a base sequence selected from the first group detects Staphylococcus aureus. An oligonucleotide probe having a base sequence selected from the second group detects Staphylococcus epidermidis. An oligonucleotide probe having a base sequence selected from the third group

detects Escherichia coli. An oligonucleotide probe having a base sequence selected from the fourth group detects Klebsiella pneumoniae. An oligonucleotide probe having a base sequence selected from the fifth group detects Pseudomonas aeruginosa. An oligonucleotide probe having a base sequence selected from the sixth group detects Serratia marcescens. An oligonucleotide probe having a base sequence selected from the seventh group detects Streptococcus pneumoniae. An oligonucleotide probe having a base sequence selected from the eighth group detects Haemophilus influenzae. An oligonucleotide probe having a base sequence selected from the ninth group detects Enterobacter cloacae. An oligonucleotide probe having a base sequence selected from the 10th group detects Enterococcus faecalis.

Complementary sequences of these probe sequences can also be used as effective probe sequences because they have the same functions as those of the above probe sequences (the complementary sequences of the first group are indicated by SEQ ID Nos. 113 to 126 in the attached sequence table, the complementary sequences of the second group are indicated by SEQ ID Nos. 127 to 136, the complementary sequences of the third group are indicated by SEQ ID Nos. 137 to 148, the complementary sequences of the fourth group are indicated by SEQ ID Nos. 149 to 159, the complementary

sequences of the fifth group are indicated by SEQ ID Nos. 160 to 169, the complementary sequences of the sixth group are indicated by SEQ ID Nos. 170 to 180, the complementary sequences of the seventh group are indicated by SEQ ID Nos. 181 to 189, the complementary sequences of the eighth group are indicated by SEQ ID Nos. 190 to 197, the complementary sequences of the ninth group are indicated by SEQ ID Nos. 198 to 209, and the complementary sequences of the 10th group are indicated by SEQ ID Nos. 210 to 218).

The probes for the respective bacteria were designed from the genome parts coding the 16s rRNAs such that they could have a very high specificity with respect to the corresponding bacteria, any variation between the probe base sequences could be prevented, and a sufficient hybridization sensitivity could be expected.

These oligonucleotide probes are designed such that a stable hybrid body is formed by hybridization reaction between a specimen and two or more kinds of probes bonded onto a carrier, and a satisfactory result can be obtained.

As a characteristic feature, the carrier according to the present invention, on which the infection detection probe of the present invention is immobilized, is prepared by discharging oligonucleotide by using a BJ printer and chemically bonding it to the

carrier. As compared to the prior arts, the probe hardly peels off. An additional effect for increasing the sensitivity is also obtained. When a DNA chip is produced by stamping called a Stanford method that is generally widely used (for example, TAKARA SHUZO produces DNA chips by applying cDNA fragments of known genes of human origin, which are related to cancers, by spotting or stamping), the applied DNA readily peels off. In addition, when a probe is laid out on a DNA chip by synthesis, as in the prior art (e.g., the DNA chip available from Affymetrix), accurate evaluation is impossible because the synthesis yield changes between probe sequences. The carrier according to the present invention is prepared also in consideration of these problems. As its characteristic features, the probe is stably immobilized and hardly peels off, as compared to the prior arts, and highly sensitive and accurate detection can be executed. The preferred embodiment of the present invention will be described below in detail.

The DNA chip of this embodiment can be applied to any specimen in which bacteria may be present, and for example, body fluids originated in animals such as human and livestock, including blood, spinal fluid, phlegm, stomach fluid, vaginal discharge, and intraoral mucus, and excretion such as urine and feces. All media which can be contaminated by bacteria can also be

subjected to a test using the DNA chip, including food, drink water and hot spring water in the natural environment, which may cause food poisoning by contamination, filters from air and water cleaners, and so forth. Animals and plants which should be quarantined in import/export are also used as specimens.

The specimens used for the DNA chip of this embodiment include not only an extracted nucleic acid itself but also specimens prepared by various methods, such as an amplified specimen prepared by using an PCR reaction primer designed for 16s rRNA detection, a specimen prepared by causing PCR reaction on the basis of a PCR amplified product, a specimen prepared by an amplification method other than PCR, and a specimen labeled by various labeling methods for visualization.

The carrier used for the DNA chip of this embodiment includes all sorts of carriers including flat substrates such as a glass substrate, a plastic substrate, and a silicon wafer, a three-dimensional structure having a three-dimensional pattern, a spherical body such as a bead, and rod-, cord-, and thread-shaped structures. The carrier also includes a substrate whose surface is processed such that a probe DNA can be immobilized. Especially, a carrier prepared by introducing a functional group to its surface to make chemical reaction possible has a preferable form

from the viewpoint of reproducibility because the probe is stably bonded in the process of hybridization reaction.

As an example of the immobilization method used in the present invention, a combination of a maleimide group and a thiol (-SH) group is used. More specifically, a thiol (-SH) group is bonded to the terminal of a nucleic acid probe, and a process is executed make the solid surface have a maleimide group. Accordingly, the thiol group of the nucleic acid probe supplied to the solid surface reacts with the maleimide group on the solid surface to immobilize the nucleic acid probe.

To introduce the maleimide group, first, an aminosilane coupling agent is caused to react on a glass substrate. Next, the maleimide group is introduced by reaction between the amino group and an EMCS reagent (N-(6-Maleimidocaproyloxy)succinimide: available from Dojin). Introduction of the SH group to a DNA can be done by using 5'-Thiol-ModifierC6 (available from Glen Research) when the DNA is synthesized by an automatic DNA synthesizer.

Instead of the above-described combination of a thiol group and a maleimide group, a combination of, e.g., an epoxy group (in the solid phase) and an amino group (nucleic acid probe terminal) can also be used as a combination of functional groups to be used for

immobilization. Surface treatments using various kinds of silane coupling agents are also effective. Oligonucleotide in which a functional group which can react with a functional group introduced by a silane coupling agent is introduced is used. A method of applying a resin having a functional group can also be used.

The present invention will be described below in more detail on the basis of examples using the infectious etiologic agent detection probes to be used to detect the 10 etiologic agents described above.

[Example 1] Microorganism Detection Using 1-Step PCR

[1. Preparation of Probe DNAs]

Nucleic acid sequences shown in Tables 1 to 10 were designed as probes to be used for detection of the 10 etiologic agents. More specifically, the following probe base sequences were selected from the genome parts coding the 16s rRNAs of the respective bacteria. These probe base sequences were designed such that they could have a very high specificity with respect to the corresponding bacteria, any variation between the probe base sequences could be prevented, and a sufficient hybridization sensitivity could be expected (The probe base sequences need not always completely match those shown in Tables 1 to 10. Probe base sequences having base lengths of 20 to 30, including the probe base sequences, are also included in the probe base sequence

shown in the tables. As described above, complementary sequences (complementary strands) of the base sequences shown in the tables may also be used).

In the following tables, "Probe No." is assigned for convenience. SEQ ID Nos. coincide with those in the attached sequence tables. As described above, the complementary strand sequences of the base sequences with SEQ ID Nos. 1 to 106 have SEQ ID Nos. 113 to 218.

Table 1 : Probes for detecting Staphylococcus aureus strain

Name of microorganizm	Probe No.	SEQ ID No.	Sequence
Staphylococcus aureus	PA-1	1	5' GAACCGCATGGTTCAAAAGTGAAAGA 3'
	PA-2	2	5' CACTTATAGATGGATCCGCGCTGC 3'
	PA-3	3	5' TGCACATCTTGACGGTACCTAATCAG 3'
	PA-4	4	5' CCCCTTAGTGCTGCAGCTAACG 3'
	PA-5	5	5' AATACAAAGGGCAGCGAAACCGC 3'
	PA-6	6	5' CCGGTGGAGTAACCTTTTAGGAGCT 3'
	PA-7	7	5' TAACCTTTTAGGAGCTAGCCGTCGA 3'
	PA-8	8	5' TTTAGGAGCTAGCCGTCGAAGGT 3'
	PA-9	9	5' TAGCCGTCGAAGGTGGGACAAAT 3'
	PA-10	10	5' ACGGACGAGAAGCTTGCTTCTCT 3'
	PA-11	11	5' TGTCACTTATAGATGGATCCGCGCT 3'
	PA-12	12	5' TGTAAGTAACTGTGCACATCTTGACG 3'
	PA-13	13	5' ACAACTCTAGAGATAGAGCCTTCCCC 3'
	PA-14	14	5' GTGGAGTAACCTTTTAGGAGCTAGCC 3'

Table 2 : Probes for detecting Staphylococcus epidermidis strain

Name of microorganizm	Probe No.	SEQ ID No.	Sequence
Staphylococcus epidermidis	PB-1	15	5' GAACAGACGAGGAGCTTGCTCC 3'
	PB-2	16	5' TAGTGAAAGACGGTTTTGCTGTCACT 3'
	PB-3	17	5' TAAGTAACTATGCACGTCTTGACGGT 3'
	PB-4	18	5' GACCCCTCTAGAGATAGAGTTTTCCC 3'
	PB-5	19	5' AGTAACCATTTGGAGCTAGCCGTC 3'
	PB-6	20	5' GAGCTTGCTCCTCTGACGTTAGC 3'
	PB-7	21	5' AGCCGGTGGAGTAACCATTTGG 3'
	PB-8	22	5' AGACGAGGAGCTTGCTCCTCTG 3'
	PB-9	23	5' AGAACAAATGTGTAAGTAACTATGCACGT 3'
	PB-10	24	5' ACCATTTGGAGCTAGCCGTCGA 3'

Table 3 : Probes for detecting Escherichia coli strain

Name of microorganizm	Probe No.	SEQ ID No.	Sequence
Escherichia coli	PC-1	25	5' CTCTTGCCATCGGATGTGCCCA 3'
	PC-2	26	5' ATACCTTTGCTCATTGACGTTACCCG 3'
	PC-3	27	5' TTTGCTCATTGACGTTACCCGCAG 3'
	PC-4	28	5' ACTGGCAAGCTTGAGTCTCGTAGA 3'
	PC-5	29	5' ATACAAAGAGAAGCGACCTCGCG 3'
	PC-6	30	5' CGGACCTCATAAAGTGCGTCGTAGT 3'
	PC-7	31	5' GCGGGGAGGAAGGGAGTAAAGTTAAT 3'
	PC-8	32	5' TAACAGGAAGAAGCTTGCTTCTTTGCTG 3'
	PC-9	33	5' TTGCCATCGGATGTGCCCAGAT 3'

	PC-10	34	5' GGAAGGGAGTAAAGTTAATACCTTTGCTC 3'
	PC-11	35	5' ATCTTTTGTTGCCAGCGGTCCG 3'
	PC-12	36	5' AAGGGAGTAAAGTTAATACCTTTGCTCATTG 3'

Table 4 : Probes for detecting Klebsiella pneumoniae strain

Name of microorganizm	Probe No.	SEQ ID No.	Sequence
Klebsiella pneumoniae	PD-1	37	5' TAGCACAGAGAGCTTGCTCTCGG 3'
	PD-2	38	5' TCATGCCATCAGATGTGCCCAGA 3'
	PD-3	39	5' CGGGGAGGAAGGCGATAAGGTTAAT 3'
	PD-4	40	5' TTCGATTGACGTTACCCGCAGAAGA 3'
	PD-5	41	5' GGTCTGTCAAGTCGGATGTGAAATCC 3'
	PD-6	42	5' GCAGGCTAGAGTCTTGTAGAGGGG 3'
	PD-7	43	5' TCATGCCATCAGATGTGCCCAGAT 3'
	PD-8	44	5' CGGGGAGGAAGGCGATAAGGTTAA 3'
	PD-9	45	5' TTATCGATTGACGTTACCCGCAGAAGA 3'
	PD-10	46	5' CATTCGAAACTGGCAGGCTAGAGTC 3'
	PD-11	47	5' CCTTTGTTGCCAGCGGTTAGGC 3'

Table 5 : Probes for detecting Pseudomonas aeruginosa strain

Name of microorganizm	Probe No.	SEQ ID No.	Sequence
Pseudomonas aeruginosa	PE-1	48	5' TGAGGGAGAAAGTGGGGGATCTTC 3'
	PE-2	49	5' TCAGATGAGCCTAGGTCGGATTAGC 3'
	PE-3	50	5' GAGCTAGAGTACGGTAGAGGGTGG 3'
	PE-4	51	5' GTACGGTAGAGGGTGGTGGAATTT 3'

	PE-5	52	5' GACCACCTGGACTGATACTGACAC 3'
	PE-6	53	5' TGGCCTTGACATGCTGAGAACTTTC 3'
	PE-7	54	5' TTAGTTACCAGCACCTCGGGTGG 3'
	PE-8	55	5' TAGTCTAACCGCAAGGGGGACG 3'
	PE-9	56	5' TGCATCCAAAACTACTGAGCTAGAGTAC 3'
	PE-10	57	5' GTCGACTAGCCGTTGGGATCCT 3'

Table 6 : Probes for detecting Serratia marcescens strain

Name of microorganizm	Probe No.	SEQ ID No.	Sequence
Serratia marcescens	PF-1	58	5' TAGCACAGGGAGCTTGCTCCCT 3'
	PF-2	59	5' AGGTGGTGAGCTTAATACGCTCATC 3'
	PF-3	60	5' TCATCAATTGACGTTACTCGCAGAAG 3'
	PF-4	61	5' ACTGCATTTGAAACTGGCAAGCTAGA 3'
	PF-5	62	5' TTATCCTTTGTTGCAGCTTCGGCC 3'
	PF-6	63	5' ACTTTCAGCGAGGAGGAAGGTGG 3'
	PF-7	64	5' GGTAGCACAGGGGAGCTTGCTC 3'
	PF-8	65	5' CGAGGAGGAAGGTGGTGAGCTTAATA 3'
	PF-9	66	5' TACGCTCATCAATTGACGTTACTCGC 3'
	PF-10	67	5' GAAACTGGCAAGCTAGAGTCTCGTAGA 3'
	PF-11	68	5' TTATCCTTTGTTGCCAGCGGTTCG 3'

Table 7 : Probes for detecting Streptococcus pneumoniae strain

Name of microorganizm	Probe No.	SEQ ID No.	Sequence
Streptococcus	PG-1	69	5' AGTAGAACGCTGAAGGAGGAGCTTG 3'

pneumoniae	PG-2	70	5' CTTGCATCACTACCAGATGGACCTG 3'
	PG-3	71	5' TGAGAGTGGAAAGTTCACACTGTGAC 3'
	PG-4	72	5' GCTGTGGCTTAACCATAGTAGGCTTT 3'
	PG-5	73	5' AAGCGGCTCTCTGGCTTGTAACT 3'
	PG-6	74	5' TAGACCCTTTCCGGGGTTTAGTGC 3'
	PG-7	75	5' GACGGCAAGCTAATCTCTTAAAGCCA 3'
	PG-8	76	5' GACATTTGCTTAAAAGGTGCACTTGCA 3'
	PG-9	77	5' GTTGTAAGAGAAGAACGAGTGTGAGAGTG 3'

Table 8 : Probes for detecting Haemophilus influenzae strain

Name of microorganizm	Probe No.	SEQ ID No.	Sequence
Haemophilus influenzae	PH-1	78	5' GCTTGGGAATCTGGCTTATGGAGG 3'
	PH-2	79	5' TGCCATAGGATGAGCCCAAGTGG 3'
	PH-3	80	5' CTTGGGAATGTACTGACGCTCATGTG 3'
	PH-4	81	5' GGATTGGGCTTAGAGCTTGGTGC 3'
	PH-5	82	5' TACAGAGGGAAGCGAAGCTGCG 3'
	PH-6	83	5' GGCGTTTACCACGGTATGATTCATGA 3'
	PH-7	84	5' AATGCCTACCAAGCCTGCGATCT 3'
	PH-8	85	5' TATCGGAAGATGAAAGTGCGGGACT 3'

Table 9 : Probes for detecting Enterobacter Cloacae strain

Name of microorganizm	Probe No.	SEQ ID No.	Sequence
Enterobacter Cloacae	PI-1	86	5' CAGAGAGCTTGCTCTCGGGTGA 3'
	PI-2	87	5' GGGAGGAAGGTGTTGTGGTTAATAAC 3'

	PI-3	88	5' GGTGTTGTGGTTAATAACCACAGCAA 3'
	PI-4	89	5' GCGGTCTGTCAAGTCGGATGTG 3'
	PI-5	90	5' ATTCGAAACTGGCAGGCTAGAGTCT 3'
	PI-6	91	5' TAACCACAGCAATTGACGTTACCCG 3'
	PI-7	92	5' GCAATTGACGTTACCCGCAGAAGA 3'
	PI-8	93	5' GTAGCACAGAGAGCTTGCTCTCG 3'
	PI-9	94	5' CGGGGAGGAAGGTGTTGTGGTTA 3'
	PI-10	95	5' ACCACAGCAATTGACGTTACCCG 3'
	PI-11	96	5' GAAACTGGCAGGCTAGAGTCTTGTAG 3'
	PI-12	97	5' AGGCGGTCTGTCAAGTCGGATG 3'

Table 10 : Probes for detecting Enterococcus faecalis strain

Name of microorganizm	Probe No.	SEQ ID No.	Sequence
Enterococcus faecalis	PJ-1	98	5' TTCTTTCCTCCCGAGTGCTTGCA 3'
	PJ-2	99	5' AACACGTGGGTAACCTACCCATCAG 3'
	PJ-3	100	5' ATGGCATAAGAGTGAAAGGCGCTT 3'
	PJ-4	101	5' GACCCGCGGTGCATTAGCTAGT 3'
	PJ-5	102	5' GGACGTTAGTAACTGAACGTCCCCT 3'
	PJ-6	103	5' CTCAACCGGGGAGGGTCATTGG 3'
	PJ-7	104	5' TTGGAGGGTTTCCGCCCTTCAG 3'
	PJ-8	105	5' ATAGAGCTTTCCCTTCGGGGACAAA 3'
	PJ-9	106	5' CGAGGTCATGCAAATCTCTTAAAGCTTCT 3'

For each probe shown in the tables, as a

functional group to immobilize the probe to a DNA microarray, a thiol group was introduced to the 5' terminal of the nucleic acid after synthesis in accordance with a conventional method. After introduction of the functional group, purification and freeze-drying were executed. The freeze-dried probes were stored in a freezer at -30°C.

[2. Preparation of Specimen Amplification PCR Primers]

As 16s rRNA gene (target gene) amplification PCR primers for etiologic agent detection, nucleic acid sequences shown in Table 11 were designed. More specifically, probe sets which specifically amplify the genome parts coding the 16s rRNAs, i.e., primers for which the specific melting points were made uniform as much as possible at the two end portions of the 16s rRNA coding region of a base length of 1,400 to 1,700 were designed. In order to simultaneously amplify variants or a plurality of 16s rRNA coding regions on genomes, a plurality of kinds of primers were designed. Note that a primer set is not limited to primer sets shown in the table 11. A primer set which is available in common to a plural kinds of etiologic agents and amplify almost entire length of 16s rRNA coding region of the etiologic agents can also be employed.

Table 11:

	Primer No.	SEQ ID No.	Sequence
Forward Primer	F-1	107	5' GCGGCGTGCCTAATACATGCAAG 3'
	F-2	108	5' GCGGCAGGCCTAACACATGCAAG 3'
	F-3	109	5' GCGGCAGGCTTAACACATGCAAG 3'
Reverse Primer	R-1	110	5' ATCCAGCCGCACCTTCCGATAC 3'
	R-2	111	5' ATCCAACCGCAGGTTCCCCTAC 3'
	R-3	112	5' ATCCAGCCGCAGGTTCCCCTAC 3'

The primers shown in Table 11 were purified by HPLC (High Performance Liquid Chromatography) after synthesis. Three forward primers and three reverse primers were mixed and dissolved in a TE buffer solution such that each primer concentration had an ultimate concentration of 10 pmol/μl.

[3. Extraction of Genome DNAs (Model Specimens) of Etiologic Agents]

[3-1] Microbial Culture & Preprocess for Genome DNA Extraction

First, microbial culture media were produced by culturing type strains of the etiologic agents (Staphylococcus aureus type strain (ATCC12600), Staphylococcus epidermidis type strain (ATCC14990), Escherichia coli type strain (ATCC11775), Klebsiella pneumoniae type strain (ATCC13883), Pseudomonas aeruginosa type strain (ATCC10145), Serratia marcescens strain, Streptococcus pneumoniae type strain,

Haemophilus influenzaestrain, Enterobacter Cloacae type strain (ATCC13047), and Enterococcus faecalis type strain (ATCC19433)) in accordance with a conventional method. Each of the microbial culture media was sampled 1.0 ml (OD_{600} = 0.7) into a 1.5-ml microtube. The cells were collected by centrifuge (8,500 rpm, 5 min, 4°C). After the supernatant was removed, a 300-µl enzyme buffer (50 mM Tris-HCl: p.H. 8.0, 25 mM EDTA) was added, and the broth was re-suspended by using a mixer. From the re-suspended broth, the cells were collected again by centrifuge (8,500 rpm, 5 min, 4°C). After the supernatant was removed, the following enzyme solution was added to the collected cells, and the broth was re-suspended by using the mixer.

Lysozyme	50 µl	(20 mg/ml in Enzyme Buffer)
N-Acetylmuramidase SG	50 µl	(0.2 mg/ml in Enzyme Buffer)

The broth added with the enzyme solution and re-suspended was left stand still in an incubator at 37°C for 30 min to melt cell walls.

[3-2] Genome Extraction

Microbial genome DNA extraction to be described below was done by using a nucleic acid purification kit (MagExtractor-Genome: available from TOYOBO).

More specifically, a 750-µl melting/absorption solution and a 40-µl magnetic beads were added to the

preprocessed microbial suspension. The suspension was intensely stirred for 10 min by using a tube mixer (step 1).

Next, the microtube was set in a separation stand (Magical Trapper) and left stand still for 30 sec to gather magnetic particles to the wall surface of the tube. The supernatant was removed while the microtube was kept set in the stand (step 2).

Next, a 900-µl cleaning solution was added. The solution was re-suspended by stirring it for about 5 sec by a mixer (step 3).

The microtube was set in the separation stand (Magical Trapper) and left stand still for 30 sec to gather magnetic particles to the wall surface of the tube. The supernatant was removed while the microtube was kept set in the stand (step 4).

Steps 3 and 4 were repeated to execute the second cleaning process (step 5). After that, 900-µl 70% ethanol was added. The solution was re-suspended by stirring it for about 5 sec by a mixer (step 6).

The microtube was set in the separation stand (Magical Trapper) and left stand still for 30 sec to gather magnetic particles to the wall surface of the tube. The supernatant was removed while the microtube was kept set in the stand (step 7).

Steps 6 and 7 were repeated to execute the second cleaning process by using 70% ethanol (step 8). After

that, 100-µl pure water was added to the collected magnetic particles. The solution was stirred for 10 min by a tube mixer (step 9).

The microtube was set in the separation stand (Magical Trapper) and left stand still for 30 sec to gather magnetic particles to the wall surface of the tube. The supernatant was collected to a new tube while the microtube was kept set in the stand.

[3-3] Test of Collected Genome DNAs

The collected genome DNAs of microorganisms (etiologic agent strain) underwent agarose electrophoresis and 260/280-nm absorbance determination in accordance with the conventional method so that the quality (the admixture amount of low molecular nucleic acid and the degree of decomposition) and collection amount were tested. In this embodiment, about 9 to 10-µg genome DNAs were collected in each bacterium. No degradation of genome DNAs or admixture of rRNA was observed. The collected genome DNAs were dissolved in a TE buffer solution at an ultimate concentration of 50 ng/µl and used in the following examples.

[4. Preparation of DNA Microarray]

The DNA Microarray was prepared according to Japanese Patent Application Laid-Open No. 11-187900.

[4-1] Cleaning of Glass Substrate

A glass substrate (size: 25 mm × 75 mm × 1 mm, available from Iiyama Tokushu Glass) made of synthetic

silica was placed in a heat- and alkali-resisting rack and dipped in a cleaning solution for ultrasonic cleaning, which was prepared to a predetermined concentration. The glass substrate was kept dipped in the cleaning solution for a night and cleaned by ultrasonic cleaning for 20 min. The substrate was picked up, lightly rinsed by pure water, and cleaned by ultrasonic cleaning in ultrapure water for 20 min. The substrate was dipped in a 1N aqueous sodium hydroxide solution heated to 80°C for 10 min. Pure water cleaning and ultrapure water cleaning were executed again. A silica glass substrate for a DNA microchip was thus prepared.

[4-2] Surface Treatment

A silane coupling agent KBM-603 (available from Shinetsu Silicone) was dissolved in pure water at a concentration of 1% and stirred at room temperature for 2 hrs. The cleaned glass substrate was dipped in the aqueous solution of the silane coupling agent and left stand still at room temperature for 20 min. The glass substrate was picked up. The surface was lightly rinsed by pure water and dried by spraying nitrogen gas to both surfaces of the substrate. The dried substrate was baked in an oven at 120°C for 1 hr to complete the coupling agent treatment, thereby introducing an amino group to the substrate surface. Next, N-(6-Maleimidocaproyloxy)succinimido) (to be

abbreviated as EMCS hereinafter) available from Dojindo Laboratories was dissolved in a 1 : 1 medium mixture of dimethyl sulfoxide and ethanol such that an ultimate concentration of 0.3 mg/ml was obtained, thereby preparing an EMCS solution.

The baked glass substrate was left stand and cooled and dipped in the prepared EMCS solution at room temperature for 2 hrs. With this process, the amino group introduced to the surface by the silane coupling agent reacted with the succinimide group in the EMCS to introduce the maleimide group to the surface of the glass substrate. The glass substrate picked up from the EMCS solution was cleaned by using the above-described medium mixture in which the EMCS was dissolved. The glass substrate was further cleaned by ethanol and dried in a nitrogen gas atmosphere.

[4-3] Probe DNA

The microorganism detection probe prepared in Example 1 was dissolved in pure water. The solution was dispensed such that the ultimate concentration (at ink dissolution) became 10 μM. Then, the solution was freeze-dried to remove water.

[4-4] DNA Discharge by BJ Printer and Bonding to Substrate

An aqueous solution containing 7.5-wt% glycerin, 7.5-wt% thioglycol, 7.5-wt% urea, and 1.0-wt% Acetylenol EH (available from Kawaken Fine Chemicals)

was prepared. Each of the seven probes (Table 1) prepared in advance was dissolved in the medium mixture at a specific concentration. An ink tank for an inkjet printer (tradename: BJF-850, available from Canon) is filled with the resultant DNA solution and attached to the printhead.

The inkjet printer used here was modified in advance to allow printing on a flat plate. When the inkjet printer inputs a printing pattern in accordance with a predetermined file creation method, an about 5-picoliter DNA solution can be spotted at a pitch of about 120 μm.

The printing operation was executed for one glass substrate by using the modified inkjet printer, thereby preparing a DNA microarray. After confirming that printing was reliably executed, the glass substrate was left stand still in a humidified chamber for 30 min to make the maleimide group on the glass substrate surface react with the thiol group at the nucleic acid probe terminal.

[4-5] Cleaning

After reaction for 30 min, the DNA solution remaining on the surface was cleaned by using a 10-mM phosphate buffer (pH 7.0) containing 100-mM NaCl, thereby obtaining a gene chip (DNA microarray) in which single-stranded DNAs were immobilized on the glass substrate surface.

[5. Amplification and Labeling of Specimens (PCR Amplification & Fluorescent Labeling)]

Amplification of microbial genes as specimens and labeling reaction will be described below

Premix PCR reagent (TAKARA ExTaq)	25 µl	
Template Genome DNA	2 µl	(100ng)
Forward Primer mix	2 µl	(20pmol/tube each)
Reverse Primer mix	2 µl	(20pmol/tube each)
Cy-3 dUTP (1mM)	2 µl	(2nmol/tube)
H_2O	17 µl	
Total	50 µl	

Amplification reaction of the reaction solution having the above composition was caused by using a commercially available thermal cycler in accordance with the following protocol.

95°C	10 min.	
92°C	45 sec.	35 Cycles
55°C	45 sec.	
72°C	45 sec.	
72°C	10 min.	

After the end of reaction, the primers were removed (purified) by using a purification column (QIAquick PCR Purification Kit available from QIAGEN).

Then, determination of the amplified products was executed to obtain labeled specimens.

[6. Hybridization]

Detection reaction was performed by using the gene chips prepared by [4. Preparation of DNA Microarray] and the labeled specimen prepared by [5. Amplification and Labeling of Specimen (PCR Amplification & Fluorescent Labeling)].

[6-1] Blocking of DNA Microarrays

BSA (fetal bovine serum albumin, Fraction V: available from Sigma) was dissolved in a 100-mM NaCl/10-mM phosphate buffer such that a 1 wt% solution was obtained. The gene chips prepared by [4. Preparation of DNA Microarray] were dipped in the solution at room temperature for 2 hrs to execute blocking. After the end of blocking, the chips were cleaned by using a 2xSSC solution (NaCl 300 mM, Sodium Citrate (trisodium citrate dihydrate, $C_6H_5Na_3 \cdot 2H_2O$) 30 mM, pH 7.0) containing 0.1-wt% SDS (Sodium Dodecyl Sulfate), rinsed by pure water, and hydro-extracted by a spin dryer.

[6-2] Hybridization

The hydro-extracted gene chips were set in a hybridization apparatus (Hybridization Station available from Genomic Solutions Inc). Hybridization reaction was caused in a hybridization solution under conditions to be described below.

[6-3] Hybridization Solution

6xSSPE/10% Form amide/Target (all 2nd PCR Products)

6xSSPE: NaCl 900 mM, $NaH_2PO_4 \cdot H_2O$ 60 mM, EDTA 6 mM, pH, 7.4)

[6-4] Hybridization Conditions

65°C 3 min → 92°C 2 min → 45°C 3 hrs → Wash 2xSSC/0.1% SDS at 25°C → Wash 2xSSC at 20°C → (Rinse with H_2O: Manual) → Spin dry (The hybridization reaction was caused at 65°C for 3 min, at 92°C for 2 min, and at 45°C for 3 hrs. The gene chips were cleaned by using 2xSSC/0.1% SDS at 25°C and 2xSSC at 20°C, rinsed by pure water, and spin-dried).

[7. Microorganism Detection (Fluorometry)]

The gene chips after the end of hybridization reaction were subjected to fluorometry by using a gene chip fluorescent detector (GenePix 4000B available from Axon). As a result, the respective bacteria could be detected with sufficient signals at a high reproducibility, as shown in Tables 12 to 21. No hybrid bodies for other bacteria were detected.

In this example, fluorometry was executed twice for each gene chip. The results are shown below.

Table 12: Staphylococcus aureus

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PA-1	5' GAACCGCATGGTTCAAAAGTGAAAGA 3'	3000	42.9	2900	40.8
PA-2	5' CACTTATAGATGGATCCGCGCTGC 3'	7700	110.0	7700	108.5
PA-3	5' TGCACATCTTGACGGTACCTAATCAG 3'	6400	91.4	6400	90.1
PA-4	5' CCCCTTAGTGCTGCAGCTAACG 3'	2500	35.7	2500	35.2
PA-5	5' AATACAAAGGGCAGCGAAACCGC 3'	7800	111.4	7800	109.9
PA-6	5' CCGGTGGAGTAACCTTTTAGGAGCT 3'	4800	68.6	4800	67.6
PA-7	5' TAACCTTTTAGGAGCTAGCCGTCGA 3'	4500	64.3	4300	60.6
PA-8	5' TTTAGGAGCTAGCCGTCGAAGGT 3'	4800	68.6	4800	67.6
PA-9	5' TAGCCGTCGAAGGTGGGACAAAT 3'	5300	75.7	5200	73.2

Table 13 : Staphylococcus epidermidis

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence uminance	S/N
PB-1	5' GAACAGACGAGGAGCTTGCTCC 3'	1000	14.5	1100	15.7
PB-2	5' TAGTGAAAGACGGTTTTGCTGTCACT 3'	1800	26.1	1800	25.7
PB-3	5' TAAGTAACTATGCACGTCTTGACGGT 3'	1400	20.3	1400	20
PB-4	5' GACCCCTCTAGAGATAGAGTTTTCCC 3'	1000	14.5	1100	15.7
PB-5	5' AGTAACCATTTGGAGCTAGCCGTC 3'	1800	26.1	2000	28.6
PB-6	5' GAGCTTGCTCCTCTGACGTTAGC 3'	1200	17.4	1300	18.6

PB-7	5' AGCCGGTGGAGTAACCATTTGG 3'	1100	15.9	1100	15.7

Table 14 : Escherichia coli

Probe No.	Sequence	First		Second	
		Fluoresc-ence luminance	S/N	Fluoresc-ence luminance	S/N
PC-1	5' CTCTTGCCATCGGATGTGCCCA 3'	1200	17.6	1200	17.9
PC-2	5' ATACCTTTGCTCATTGACGTTACCCG 3'	1500	22.1	1600	23.9
PC-3	5' TTTGCTCATTGACGTTACCCGCAG 3'	1100	16.2	1200	17.9
PC-4	5' ACTGGCAAGCTTGAGTCTCGTAGA 3'	2000	29.4	2100	31.3
PC-5	5' ATACAAAGAGAAGCGACCTCGCG 3'	1500	22.1	1500	22.4
PC-6	5' CGGACCTCATAAAGTGCGTCGTAGT 3'	2400	35.3	2600	38.8
PC-7	5' GCGGGGAGGAAGGGAGTAAAGTTAAT 3'	1200	17.6	1200	17.9

Table 15 : Klebsiella pneumoniae

Probe No.	Sequence	First		Second	
		Fluoresc-Ence luminance	S/N	Fluoresc-ence luminance	S/N
PD-1	5' TAGCACAGAGAGCTTGCTCTCGG 3'	500	7.6	600	9
PD-2	5' TCATGCCATCAGATGTGCCCAGA 3'	600	9.1	600	9
PD-3	5' CGGGGAGGAAGGCGATAAGGTTAAT 3'	700	10.6	700	10.4
PD-4	5' TTCGATTGACGTTACCCGCAGAAGA 3'	1000	15.2	1200	17.9
PD-5	5' GGTCTGTCAAGTCGGATGTGAAATCC 3'	2700	40.9	2700	40.3
PD-6	5' GCAGGCTAGAGTCTTGTAGAGGGG 3'	3400	51.5	3300	49.3

Table 16 : Pseudomonas aeruginosa

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PE-1	5' TGAGGGAGAAAGTGGGGGATCTTC 3'	3500	50.0	3600	50
PE-2	5' TCAGATGAGCCTAGGTCGGATTAGC 3'	1600	22.9	1400	19.4
PE-3	5' GAGCTAGAGTACGGTAGAGGGTGG 3'	3500	50.0	3400	47.2
PE-4	5' GTACGGTAGAGGGTGGTGGAATTT 3'	3100	44.3	3100	43.1
PE-5	5' GACCACCTGGACTGATACTGACAC 3'	1600	22.9	1600	22.2
PE-6	5' TGGCCTTGACATGCTGAGAACTTTC 3'	1200	17.1	1200	16.7
PE-7	5' TTAGTTACCAGCACCTCGGGTGG 3'	1000	14.3	1200	16.7
PE-8	5' TAGTCTAACCGCAAGGGGGACG 3'	1100	15.7	1100	15.3

Table 17 : Serratia marcescens

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PF-1	5' TAGCACAGGGAGCTTGCTCCCT 3'	600	8.8	600	8.7
PF-2	5' AGGTGGTGAGCTTAATACGCTCATC 3'	700	10.3	600	8.7
PF-3	5' TCATCAATTGACGTTACTCGCAGAAG 3'	2000	29.4	2200	31.9
PF-4	5' ACTGCATTTGAAACTGGCAAGCTAGA 3'	2800	41.2	2700	39.1
PF-5	5' TTATCCTTTGTTGCAGCTTCGGCC 3'	700	10.3	700	10.1
PF-6	5' ACTTTCAGCGAGGAGGAAGGTGG 3'	3400	50.0	3300	47.8

Table 18 : Streptococcus pneumoniae

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PG-1	5' AGTAGAACGCTGAAGGAGGAGCTTG 3'	1000	14.9	1100	16.2
PG-2	5' CTTGCATCACTACCAGATGGACCTG 3'	1200	17.9	1300	19.1
PG-3	5' TGAGAGTGGAAAGTTCACACTGTGAC 3'	1000	14.9	1100	16.2
PG-4	5' GCTGTGGCTTAACCATAGTAGGCTTT 3'	1800	26.9	1900	27.9
PG-5	5' AAGCGGCTCTCTGGCTTGTAACT 3'	1300	19.4	1500	22.1
PG-6	5' TAGACCCTTTCCGGGGTTTAGTGC 3'	1300	19.4	1300	19.1
PG-7	5' GACGGCAAGCTAATCTCTTAAAGCCA 3'	2000	29.9	2100	30.9

Table 19 : Haemophilus influenzae

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PH-1	5' GCTTGGGAATCTGGCTTATGGAGG 3'	3500	50.0	3600	50
PH-2	5' TGCCATAGGATGAGCCCAAGTGG 3'	600	8.8	700	10.1
PH-3	5' CTTGGGAATGTACTGACGCTCATGTG 3'	600	8.8	600	8.7
PH-4	5' GGATTGGGCTTAGAGCTTGGTGC 3'	1100	16.2	1200	17.4
PH-5	5' TACAGAGGGAAGCGAAGCTGCG 3'	700	10.3	600	8.7
PH-6	5' GGCGTTTACCACGGTATGATTCATGA 3'	1300	19.1	1300	18.8
PH-7	5' AATGCCTACCAAGCCTGCGATCT 3'	2100	30.9	2200	31.9

PH-8	5' TATCGGAAGATGAAAGTGCGGGACT 3'	700	10.3	600	8.7

Table 20 : Enterobacter cloacae

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PI-1	5' CAGAGAGCTTGCTCTCGGGTGA 3'	2100	29.2	2200	31
PI-2	5' GGGAGGAAGGTGTTGTGGTTAATAAC 3'	7900	109.7	7900	111.3
PI-3	5' GGTGTTGTGGTTAATAACCACAGCAA 3'	1000	13.9	1300	18.3
PI-4	5' GCGGTCTGTCAAGTCGGATGTG 3'	6400	88.9	6400	90.1
PI-5	5' ATTCGAAACTGGCAGGCTAGAGTCT 3'	9400	130.6	9200	129.6
PI-6	5' TAACCACAGCAATTGACGTTACCCG 3'	4700	65.3	4800	67.6
PI-7	5' GCAATTGACGTTACCCGCAGAAGA 3'	4600	63.9	4500	63.4

Table 21 : Enterococcus faecalis

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PJ-1	5'TTCTTTCCTCCCGAGTGCTTGCA 3'	1500	22.1	1500	20.8
PJ-2	5'AACACGTGGGTAACCTACCCATCAG 3'	2400	35.3	2700	37.5
PJ-3	5'ATGGCATAAGAGTGAAAGGCGCTT 3'	5600	82.4	5600	77.8
PJ-4	5'GACCCGCGGTGCATTAGCTAGT 3'	2300	33.8	2300	31.9
PJ-5	5'GGACGTTAGTAACTGAACGTCCCCT 3'	1000	14.7	1400	19.4
PJ-6	5'CTCAACCGGGGAGGGTCATTGG 3'	4400	64.7	4400	61.1

PJ-7	5'TTGGAGGGTTTCCGCCCTTCAG 3'	1700	25	1800	25

The numerical values of florescent luminances(photomultiplier voltage: 400 V) in Tables 12 to 21 indicate average pixel luminances (resolution: 5 μm). The S/N ratios indicate values obtained by dividing the fluorescent luminances by background average values measured by analysis software (GenePix Pro Ver.3.0 available from Axon) attached to the measuring device.

As is apparent from Tables 12 to 21, the respective etiologic agents can be detected with sufficient signals at a high reproducibility.

[Example 2] Microorganism Detection Using 2-Step PCR

As in Example 1, probe DNAs, specimen amplification PCR primers, the genome DNAs of etiologic agents, and DNA microarrays were prepared, and the following experiments were conducted.

[1. Amplification and Labeling of Specimens (PCR Amplification & Fluorescent Labeling)]

Amplification of microbial genes as specimens (1st PCR) and labeling (2nd PCR) reaction will be described below.

[2. Amplification Reaction Solution Composition: 1st PCR]

Premix PCR reagent (TAKARA 25 μl

ExTaq)		
Template Genome DNA	2 µl	(10ng)
Forward Primer mix	2 µl	(20pmol/tube each)
Reverse Primer mix	2 µl	(20pmol/tube each)
H_2O	19 µl	
Total	50 µl	

Amplification reaction of the reaction solution having the above composition was caused by using a commercially available thermal cycler in accordance with the following protocol.

95°C	10 min.
92°C	45 sec.
55°C	45 sec.
72°C	45 sec.
72°C	10 min.

25 Cycles

After the end of reaction, purification was performed by using a purification column (QIAquick PCR Purification Kit available from QIAGEN). Then, determination of the amplified products was executed.

[3. Labeling Reaction Solution Composition: 2nd PCR]

Enzyme (QIAGEN Hotstar Taq Polymerase)	0.5 µl	(2.5u)
Template DNA (1st PCR Product)	10 µl	(30ng)

dNTP mix (Low dTTP)*	2 µl	
Cy-3 dUTP (1mM)	2 µl	(2nmol/tube)
Reverse Primer mix	5 µl	(50pmol/tube each)
10 x Buffer	5 µl	
H_2O	25.5 µl	
Total	50 µl	

*dNTP mix (Low dTTP) :

dATP,dCTP,dGTP / 5mM(final : 10 nmol/tube)

dTTP / 4mM (final : 8 nmol/tube)

Amplification reaction of the reaction solution having the above composition was caused by using a commercially available thermal cycler in accordance with the following protocol.

95°C	10 min.	
92°C	45 sec.	25 Cycles
55°C	45 sec.	
72°C	45 sec.	
72°C	10 min.	

After the end of reaction, purification was performed by using a purification column (QIAquick PCR Purification Kit available from QIAGEN) to obtain labeled specimens.

[4. Hybridization]

Hybridization was done in accordance with the same procedures as in Example 1.

[5. Microorganism Detection (Fluorometry)]

Fluorometry was executed for the DNA microarrays after the end of hybridization reaction by using a DNA microarray fluorescent detector (GenePix 4000B available from Axon). Tables 22 to 31 show the measurement results.

Even in this example, fluorometry was executed twice for each DNA microarray. The results are shown in Tables 22 to 31.

Table 22: Staphylococcus aureus

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PA-1	5' GAACCGCATGGTTCAAAAGTGAAAGA 3'	14000	186.7	13000	173.3
PA-2	5' CACTTATAGATGGATCCGCGCTGC 3'	36000	480	35000	466.7
PA-3	5' TGCACATCTTGACGGTACCTAATCAG 3'	31000	413.3	29000	386.7
PA-4	5' CCCCTTAGTGCTGCAGCTAACG 3'	10000	133.3	10000	133.3
PA-5	5' AATACAAAGGGCAGCGAAACCGC 3'	39000	520	38500	513.3
PA-6	5' CCGGTGGAGTAACCTTTTAGGAGCT 3'	22000	293.3	22100	294.7
PA-7	5' TAACCTTTTAGGAGCTAGCCGTCGA 3'	22000	293.3	21800	290.7
PA-8	5' TTTAGGAGCTAGCCGTCGAAGGT 3'	25000	333.3	24000	320
PA-9	5' TAGCCGTCGAAGGTGGGACAAAT 3'	26000	346.7	25500	340

Table 23 : Staphylococcus epidermidis

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PB-1	5' GAACAGACGAGGAGCTTGCTCC 3'	4500	62.5	4700	67.1
PB-2	5' TAGTGAAAGACGGTTTTGCTGTCACT 3'	9000	125	8900	127.1
PB-3	5' TAAGTAACTATGCACGTCTTGACGGT 3'	7100	98.6	7300	104.3
PB-4	5' GACCCCTCTAGAGATAGAGTTTTCCC 3'	4800	66.7	5200	74.3
PB-5	5' AGTAACCATTTGGAGCTAGCCGTC 3'	9100	126.4	9300	132.9
PB-6	5' GAGCTTGCTCCTCTGACGTTAGC 3'	5800	80.6	6300	90
PB-7	5' AGCCGGTGGAGTAACCATTTGG 3'	5400	75	5500	78.6

Table 24 : Escherichia coli

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PC-1	5' CTCTTGCCATCGGATGTGCCCA 3'	5600	76.7	6200	83.8
PC-2	5' ATACCTTTGCTCATTGACGTTACCCG 3'	7600	104.1	7500	101.4
PC-3	5' TTTGCTCATTGACGTTACCCGCAG 3'	5600	76.7	5700	77
PC-4	5' ACTGGCAAGCTTGAGTCTCGTAGA 3'	9400	128.8	9300	125.7
PC-5	5' ATACAAAGAGAAGCGACCTCGCG 3'	7200	98.6	7200	97.3
PC-6	5' CGGACCTCATAAAGTGCGTCGTAGT 3'	11500	157.5	11500	155.4
PC-7	5' GCGGGGAGGAAGGGAGTAAAGTTAAT 3'	5600	76.7	5500	74.3

Table 25 : Klebsiella pneumoniae

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PD-1	5' TAGCACAGAGAGCTTGCTCTCGG 3'	2000	28.6	2100	30
PD-2	5' TCATGCCATCAGATGTGCCCAGA 3'	2500	35.7	2600	37.1
PD-3	5' CGGGGAGGAAGGCGATAAGGTTAAT 3'	2900	41.4	2900	41.4
PD-4	5' TTCGATTGACGTTACCCGCAGAAGA 3'	4500	64.3	4700	67.1
PD-5	5' GGTCTGTCAAGTCGGATGTGAAATCC 3'	9900	141.4	10100	144.3
PD-6	5' GCAGGCTAGAGTCTTGTAGAGGGG 3'	13000	185.7	13400	191.4

Table 26 : Pseudomonas aeruginosa

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PE-1	5' TGAGGGAGAAAGTGGGGGATCTTC 3'	17000	239.4	17300	240.3
PE-2	5' TCAGATGAGCCTAGGTCGGATTAGC 3'	8300	116.9	8600	119.4
PE-3	5' GAGCTAGAGTACGGTAGAGGGTGG 3'	17400	245.1	17000	236.1
PE-4	5' GTACGGTAGAGGGTGGTGGAATTTC 3'	15000	211.3	16000	222.2
PE-5	5' GACCACCTGGACTGATACTGACAC 3'	8000	112.7	8300	115.3
PE-6	5' TGGCCTTGACATGCTGAGAACTTTC 3'	5400	76.1	5800	80.6
PE-7	5' TTAGTTACCAGCACCTCGGGTGG 3'	5300	74.6	5100	70.8
PE-8	5' TAGTCTAACCGCAAGGGGGACG 3'	5400	76.1	5000	69.4

Table 27 : Serratia marcescens

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PF-1	5' TAGCACAGGGAGCTTGCTCCCT 3'	3100	43.7	3300	45.2
PF-2	5' AGGTGGTGAGCTTAATACGCTCATC 3'	3300	46.5	3200	43.8
PF-3	5' TCATCAATTGACGTTACTCGCAGAAG 3'	10100	142.3	10000	137
PF-4	5' ACTGCATTTGAAACTGGCAAGCTAGA 3'	12000	169	11800	161.6
PF-5	5' TTATCCTTTGTTGCAGCTTCGGCC 3'	4100	57.7	4200	57.5
PF-6	5' ACTTTCAGCGAGGAGGAAGGTGG 3'	14300	201.4	14300	195.9

Table 28 : Streptococcus pneumoniae

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PG-1	5' AGTAGAACGCTGAAGGAGGAGCTTG 3'	4500	63.4	4300	60.6
PG-2	5' CTTGCATCACTACCAGATGGACCTG 3'	5800	81.7	5600	78.9
PG-3	5' TGAGAGTGGAAAGTTCACACTGTGAC 3'	5000	70.4	4900	69
PG-4	5' GCTGTGGCTTAACCATAGTAGGCTTT 3'	8700	122.5	8800	123.9
PG-5	5' AAGCGGCTCTCTGGCTTGTAACT 3'	7200	101.4	7300	102.8
PG-6	5' TAGACCCTTTCCGGGGTTTAGTGC 3'	6700	94.4	7000	98.6
PG-7	5' GACGGCAAGCTAATCTCTTAAAGCCA 3'	10200	143.7	9900	139.4

Table 29 : Haemophilus influenzae

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PH-1	5'GCTTGGGAATCTGGCTTATGGAGG 3'	3100	44.3	3200	45.1
PH-2	5'TGCCATAGGATGAGCCCAAGTGG 3'	3200	45.7	3200	45.1
PH-3	5'CTTGGGAATGTACTGACGCTCATGTG 3'	4900	70	5600	78.9
PH-4	5'GGATTGGGCTTAGAGCTTGGTGC 3'	3900	55.7	3800	53.5
PH-5	5'TACAGAGGGAAGCGAAGCTGCG 3'	6700	95.7	6500	91.5
PH-6	5'GGCGTTTACCACGGTATGATTCATGA 3'	10200	145.7	11000	154.9
PH-7	5'AATGCCTACCAAGCCTGCGATCT 3'	4200	60	4100	57.7
PH-8	5'TATCGGAAGATGAAAGTGCGGGACT 3'	3200	45.7	3500	49.3

Table 30 : Enterobacter cloacae

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PI-1	5' CAGAGAGCTTGCTCTCGGGTGA 3'	10000	133.3	9900	133.8
PI-2	5'GGGAGGAAGGTGTTGTGGTTAATAAC 3'	38000	506.7	38000	513.5
PI-3	5'GGTGTTGTGGTTAATAACCACAGCAA 3'	4700	62.7	4700	63.5
PI-4	5'GCGGTCTGTCAAGTCGGATGTG 3'	31000	413.3	32000	432.4
PI-5	5'ATTCGAAACTGGCAGGCTAGAGTCT 3'	47500	633.3	45000	608.1
PI-6	5'TAACCACAGCAATTGACGTTACCCG 3'	23600	314.7	24000	324.3
PI-7	5'GCAATTGACGTTACCCGCAGAAGA 3'	21500	286.7	22700	306.8

Table 31 : Enterococcus faecalis

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PJ-1	5'TTCTTTCCTCCCGAGTGCTTGCA 3'	7000	98.6	7300	101.4
PJ-2	5'AACACGTGGGTAACCTACCCATCAG 3'	12300	173.2	12000	166.7
PJ-3	5'ATGGCATAAGAGTGAAAGGCGCTT 3'	25000	352.1	27400	380.6
PJ-4	5'GACCCGCGGTGCATTAGCTAGT 3'	10000	140.8	11000	152.8
PJ-5	5'GGACGTTAGTAACTGAACGTCCCCT 3'	5600	78.9	5200	72.2
PJ-6	5'CTCAACCGGGGAGGGTCATTGG 3'	22100	311.3	22200	308.3
PJ-7	5'TTGGAGGGTTTCCGCCCTTCAG 3'	8800	123.9	9000	125

The numerical values of florescent luminances(photomultiplier voltage: 400 V) in Tables 22 to 31 indicate average pixel luminances (resolution: 5 μm). The S/N ratios indicate values obtained by dividing the fluorescent luminances by background average values measured by analysis software (GenePix Pro Ver.3.0 available from Axon) attached to the measuring device.

As is apparent from Tables 22 to 31, the respective etiologic agents can be detected with sufficient signals at a high reproducibility.

[Example 3] Microorganism Detection Using 2-Step PCR

As in Examples 1 and 2, probe DNAs, specimen amplification PCR primers, the genome DNAs of etiologic agents, and DNA microarrays were prepared, and the following experiments were conducted.

[1. Amplification and Labeling of Specimens (Utilization of PCR Amplification with Fluorescent Labeling)]

Amplification of microbial genes as specimens (1st PCR) and labeling (2nd PCR) reaction will be described below.

[2. Amplification Reaction Solution Composition: 1st PCR]

AmpliTaq Gold LD(5U/μL)	0.5μL
Template DNA	variable
dNTP mis(2.5mM/each)	4.0μL
x10 PCR buffer	5.0μL
25mM $MgCl_2$	7.0μL
Forward Primer Mix(10μM/each)	0.25μL
Reverse Primer Mix(10μM/each)	0.25μL
H_2O	variable
Total	50μL

Amplification reaction of the reaction solution having the above composition was caused by using a

commercially available thermal cycler in accordance with the following protocol.

95°C	10 min.	
92°C	45 sec.	39 Cycles
67°C	45 sec.	
72°C	45 sec.	
72°C	10 min.	

After the end of reaction, purification was performed by using a purification column (QIAquick PCR Purification Kit available from QIAGEN). Then, determination of the amplified products was executed.

[3. Labeling Reaction Composition: 2nd PCR]

Premix PCR reagent(TAKARA ExTaq)	25 µl
Template DNA (1st PCR Product)	Variable (30ng/tube)
Cy3 Labeled Reverse primer Mix	5 µl
H_2O	Variable
Total	50 µl

Amplification reaction of the reaction solution having the above composition was caused by using a commercially available thermal cycler in accordance with the following protocol.

95°C	10 min.	
92°C	45 sec.	24 Cycles
65°C	45 sec.	
72°C	45 sec.	
72°C	10 min.	

After the end of reaction, purification was performed by using a purification column (QIAquick PCR Purification Kit available from QIAGEN) to obtain labeled specimens.

[4. Hybridization]

Hybridization was done in accordance with the same procedures as in Example 1.

[5. Microorganism Detection (Fluorometry)]

Fluorometry was executed for the DNA microarrays after the end of hybridization reaction by using a DNA microarray fluorescent detector (GenePix 4000B available from Axon). Tables 32 to 41 show the measurement results.

Note that, in this example, fluorometry was executed once twice for each DNA microarray. The results are shown in Tables 32 to 41.

Table 32: Staphylococcus aureus

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PA-10	5' ACGGACGAGAAGCTTGCTTCTCT 3'	247	3.4	146	2.1
PA-11	5' TGTCACTTATAGATGGATCCGCGCT 3'	4177	57.9	3083	43.4
PA-12	5' TGTAAGTAACTGTGCACATCTTGACG 3'	4686	64.9	3768	53.1
PA-13	5' ACAACTCTAGAGATAGAGCCTTCCCC 3'	2612	36.2	2709	38.2
PA-14	5' GTGGAGTAACCTTTTAGGAGCTAGCC 3'	26505	367.2	17560	247.3

Table 33 : Staphylococcus epidermidis

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PB-2	5' TAGTGAAAGACGGTTTTGCTGTCACT 3'	7000	94.1	1800	25.7
PB-4	5' GACCCCTCTAGAGATAGAGTTTTCCC 3'	3274	44.0	1100	15.7
PB-8	5' AGACGAGGAGCTTGCTCCTCTG 3'	111	1.5	59	0.8
PB-9	5' AGAACAAATGTGTAAGTAACTATGCACGT 3'	6920	93.0	4910	70.1
PB-10	5' ACCATTTGGAGCTAGCCGTCGA 3'	15244	205.0	18136	259.1

Table 34 : Escherichia coli

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PC-4	5' ACTGGCAAGCTTGAGTCTCGTAGA 3'	5416	74.7	2100	31.3
PC-8	5' TAACAGGAAGAAGCTTGCTTCTTTGCTG 3'	160	2.2	112	1.7
PC-9	5' TTGCCATCGGATGTGCCCAGAT 3'	4133	57.0	4581	68.4
PC-10	5' GGAAGGGAGTAAAGTTAATACCTTTGCTC 3'	4194	57.8	5349	79.8
PC-11	5' ATCTTTTGTTGCCAGCGGTCCG 3'	6719	92.7	2594	38.7
PC-12	5' AAGGGAGTAAAGTTAATACCTTTGCTCATTG 3'	3984	58.6	4021	60.0

Table 35 : Klebsiella pneumoniae

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PD-7	5' TCATGCCATCAGATGTGCCCAGAT 3'	5414	40.0	4171	62.3
PD-8	5' CGGGGAGGAAGGCGATAAGGTTAA 3'	4096	30.2	6227	93.0
PD-9	5' TTATCGATTGACGTTACCCGCAGAAGA 3'	4122	30.4	3269	48.8
PD-10	5' CATTCGAAACTGGCAGGCTAGAGTC 3'	9474	70.0	6486	96.9
PD-11	5' CCTTTGTTGCCAGCGGTTAGGC 3'	10648	78.6	2754	41.1

Table 36 : Pseudomonas aeruginosa

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PE-1	5' TGAGGGAGAAAGTGGGGGATCTTC 3'	6175	82.2	3600	50.0
PE-6	5' TGGCCTTGACATGCTGAGAACTTTC 3'	8159	108.6	1200	16.7
PE-7	5' TTAGTTACCAGCACCTCGGGTGG 3'	3277	43.6	1200	16.7
PE-9	5' TGCATCCAAAACTACTGAGCTAGAGTAC 3'	6626	88.2	7432	103.4
PE-10	5' GTCGACTAGCCGTTGGGATCCT 3'	5734	76.3	3365	46.8

Table 37 : Serratia marcescens

	Probe No.	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PF-7	5' GGTAGCACAGGGGAGCTTGCTC 3'	4482	66.4	1040	15.1
PF-8	5' CGAGGAGGAAGGTGGTGAGCTTAATA 3'	6362	94.2	3199	46.3
PF-9	5' TACGCTCATCAATTGACGTTACTCGC 3'	4569	67.7	2884	41.8
PF-10	5' GAAACTGGCAAGCTAGAGTCTCGTAGA 3'	7905	117.1	6786	98.3
PF-11	5' TTATCCTTTGTTGCCAGCGGTTCG 3'	12787	189.4	3849	55.7

Table 38 : Streptococcus pneumoniae

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PG-1	5' AGTAGAACGCTGAAGGAGGAGCTTG 3'	10078	70.3	1100	16.2
PG-5	5' AAGCGGCTCTCTGGCTTGTAACT 3'	4331	30.2	1500	22.1
PG-6	5' TAGACCCTTTCCGGGGTTTAGTGC 3'	4730	33.0	1300	19.1
PG-8	5' GACATTTGCTTAAAAGGTGCACTTGCA 3'	7128	49.7	7720	113.6
PG-9	5' GTTGTAAGAGAAGAACGAGTGTGAGAGTG 3'	6665	46.5	3297	48.5

Table 39 : Haemophilus influenzae

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PH-1	5' GCTTGGGAATCTGGCTTATGGAGG 3'	11106	150.3	3600	50.0
PH-2	5' TGCCATAGGATGAGCCCAAGTGG 3'	7056	95.5	700	10.1
PH-4	5' GGATTGGGCTTAGAGCTTGGTGC 3'	100	1.4	1200	17.4
PH-5	5' TACAGAGGGAAGCGAAGCTGCG 3'	11237	152.1	600	8.7
PH-7	5' AATGCCTACCAAGCCTGCGATCT 3'	5054	68.4	2200	31.9

Table 40 : Enterobacter cloacae

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PI-8	5' GTAGCACAGAGAGCTTGCTCTCG 3'	2221	30.1	582	8.2
PI-9	5' CGGGGAGGAAGGTGTTGTGGTTA 3'	5484	74.2	2193	30.9
PI-10	5' ACCACAGCAATTGACGTTACCCG 3'	3325	45.0	646	9.1
PI-11	5' GAAACTGGCAGGCTAGAGTCTTGTAG 3'	7574	102.5	3039	42.8
PI-12	5' AGGCGGTCTGTCAAGTCGGATG 3'	5768	78.0	5701	80.3

Table 41 : Enterococcus faecalis

Probe No.	Sequence	First		Second	
		Fluorescence luminance	S/N	Fluorescence luminance	S/N
PJ-1	5' TTCTTTCCTCCCGAGTGCTTGCA 3'	1012	14.9	1500	20.8
PJ-3	5' ATGGCATAAGAGTGAAAGGCGCTT 3'	4266	62.6	5600	77.8
PJ-5	5' GGACGTTAGTAACTGAACGTCCCCT 3'	652	9.6	1400	19.4
PJ-8	5' ATAGAGCTTTCCCTTCGGGGACAAA 3'	3232	47.5	810	11.2
PJ-9	5' CGAGGTCATGCAAATCTCTTAAAGCTTCT 3'	11411	167.6	18776	260.7

As is apparent from Tables 32 to 41, the respective etiologic agents can be detected with sufficient signals at a high reproducibility.

As described above, according to the examples, an

infectious etiologic agent can be identified by using microarrays on which probe sets capable of detecting the 10 bacteria, i.e., Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens, Streptococcus pneumoniae, Haemophilus influenzae, Enterobacter cloacae, and Enterococcus faecalis are separately immobilized or combined. Hence, the problems of the DNA probe of microbial origin are solved. More specifically, because of the small number of bases, oligonucleotide probes can chemically be mass-produced, and purification and concentration control are possible. In addition, a probe set can be provided, which allows to detect bacteria in the same species all together and discriminatingly detect bacteria in other species for the purpose of classifying the bacteria depending on the species.

Furthermore, a probe set can be provided, which also considers the stability of a hybrid body between a probe and a specimen so that the difference between the species can accurately be evaluated on a DNA microarray. A carrier on which the probe DNAs are immobilized to make the probe DNAs react with specimens can also be provided. Also, a carrier can be provided, on which the probe DNAs are chemically immobilized so that the probe DNAs are stably immobilized on the

carrier, and a detection result with high reproducibility can be obtained in the process of reaction between a specimen solution and the probes and probe sets.

According to the above examples, 16s rRNA gene arrangements in the genes of infectious etiologic agents can be detected in proper quantities. Hence, the presence of an infectious etiologic agent can efficiently and accurately be determined.

[Example 4] Primer Set

The primer sets (Table 11) used in the above examples to amplify the 16s rRNA gene arrangements of one or some of Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens, Streptococcus pneumoniae, Haemophilus influenzae, Enterobacter cloacae, and Enterococcus faecalis will be described.

The primer sets of this example are designed to give a satisfactory amplification result in PCR reaction executed to identify an infectious etiologic agent. "Satisfactory" means not only that the target 16s rRNAs are sufficiently amplified but also that no products other than the 16s rRNAs are generated.

"Satisfactory" also means that only the 16s rRNAs of an infectious etiologic agent are amplified without

amplifying human genome genes originated in a specimen, which are contained in the specimen.

Any specimen in which bacteria may be present, and for example, body fluids originated in animals such as human and livestock, including blood, spinal fluid, phlegm, stomach fluid, vaginal discharge, and intraoral mucus, and excretion such as urine and feces are used in this example. All media which can be contaminated by bacteria can also be used in this example, including food, drink water and hot spring water in the natulal environment, which may cause food poisoning by contamination, filters from air and water cleaners, and so forth. Animals and plants which should be quarantined in import/export are also used as specimens.

The PCR reaction used in this example includes PCR reaction which uses an extracted nucleic acid itself as a template, asymmetrical PCR reaction which uses primers on one side of SEQ ID Nos. 107 to 109 (F1 to F3 in Table 11) or SEQ ID Nos. 110 to 112 (R1 to R3 in Table 11), and PCR which executes labeling for visualization.

[1. Preparation of Specimen Amplification PCR Primers]

As 16s rRNA gene (target gene) amplification PCR primers for etiologic agent detection, nucleic acid sequences shown in Table 11 were designed.

More specifically, probe sets which specifically

amplify the genome parts coding the 16s rRNAs, i.e., primers for which the specific melting points were made uniform as much as possible at the two end portions of the 16s rRNA coding region of a base length of 1,500 were designed. In order to simultaneously amplify variants or a plurality of 16s rRNA coding regions on genomes, a plurality of kinds of primers were designed.

The primers shown in Table 11 were purified by HPLC (High Performance Liquid Chromatography) after synthesis. All of three forward primers and three reverse primers were mixed and dissolved in a TE buffer solution such that each primer concentration had an ultimate concentration of 10 pmol/μl. In this example, all the forward primers and reverse primers were used. Alternatively, one to three forward primers and one to three reverse primers may be used.

By using a thus prepared solution of forward primers and reverse primers (forward primer mix and reverse primer mix), genome DNAs extracted by the method described in [3. Extraction of Genome DNAs (Model Specimens) of Etiologic Agents] were amplified by the method described in [5. Amplification and Labeling of Specimens (PCR Amplification & Fluorescent Labeling)].

After the end of reaction, the primers were removed by using a purification column (QIAquick PCR Purification Kit available from QIAGEN). Then, the

amplified products were examined by gel electrophoresis. One band was detected in 1,500 base pair regions, and it was confirmed that satisfactory PCR reaction was executed. No byproducts were generated.

When the primers shown in Table 11 were used, satisfactory PCR amplification results were obtained in, e.g., all of the above-described 10 infectious etiologic agents (Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens, Streptococcus pneumoniae, Haemophilus influenzae, Enterobacter cloacae, and Enterococcus faecalis).

[Example 5] Amplification of 16s rRNA Genes from Mixture of Blood and Broth

Bacteremia model systems were prepared by adding 10^3, 10^4, and 10^5 Enterobacter cloacae, which was cultured in accordance with the procedures described in Example 1, to 200-µl human blood (collected EDTA blood). An N-acetyl muramidase solution (0.2 mg/ml in Enzyme Buffer) was added to each solution. The solutions were heated to 37°C for 30 min. After that, DNAs were extracted by using Qiamp Blood mini Kit (available from QIAGEN) to prepare templates for PCR reaction.

PCR reaction was caused for these DNAs by using the primers shown in Table 11, as in Example 4.

As a result, as in Example 4, one band was detected in 1,500 base pair regions, and it was confirmed that satisfactory PCR reaction was executed. No byproducts were generated. The amount of PCR amplified products obtained from the band was proportional to the added cell amount. This indicates that when the primer sets were used, only the 16s rRNAs of Enterobacter cloacae were amplified without generating any PCR byproduct of human genome.

As described above, according to this example, the 16s rRNA parts in the genes of a plurality of kinds of infectious etiologic agents can efficiently be amplified at a high purity. In addition, even when human genome DNAs are present, only the 16s rRNAs of an infectious etiologic agent can efficiently be amplified.

As has been described above, according to the present invention, an infection detection probe which allows mass preparation at a time and identification of a species in similar species can be provided. More specifically, an infection detection probe which can suitably be used to classify a plurality of kinds of etiologic agents of an infection on the basis of the species can be provided.

Alternatively, an infection detection probe suitable for detection of, e.g., the above-described 10 bacteria as the etiologic agents of infections can be

provided.

A probe set can also be provided, which also considers the stability of a hybrid body between an infection detection probe and a specimen so that the difference between similar species can accurately be evaluated on a DNA chip.

In addition, a carrier on which the infection detection probe is immobilized to make the infection detection probe react with the specimen can be provided.

Furthermore, a carrier can be provided, on which the infection detection probes are chemically immobilized so that the infection detection probes are stably immobilized on the carrier, and a detection result with high reproducibility can be obtained in the process of reaction with a specimen solution.

According to the present invention, there is also provided a PCR reaction primer which amplifies the 16s rRNAs of an etiologic agent in a specimen in order to detect and/or identify an infectious etiologic agent.

According to the present invention, there is also provided a primer set which can commonly be used for a plurality of species and effectively amplify the 16s rRNAs of an etiologic agent even when the species is unknown.

According to the present invention, there is also provided a primer set which can amplify the 16s rRNAs

of a plurality of kinds of etiologic agents under the same PCR conditions.

As many apparently widely different embodiments of the present invention can be made without departing from the spirit and scope thereof, it is to be understood that the invention is not limited to the specific embodiments thereof except as defined in the appended claims.